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14. ABSTRACT

Tuberous sclerosis complex (TSC) is an autosomal disorder resulting from mutations in the TSC1 or TSC2 genes that is associated with epilepsy, cognitive disability, and autism. TSC1/TSC2 gene mutations lead to developmental alterations in brain structure known as tubers in over 80% of TSC patients. Loss of TSC1 or TSC2 function in tubers results from biallelic TSC gene inactivation and leads to activation of the mTOR cascade as evidenced by phosphorylation of ribosomal S6 protein (P-S6). We demonstrate that there are numerous cytoarchitectural abnormalities in non-tuber brain areas in post-mortem TSC brain. Many of these regions exhibit aberrant phosphorylation of the ribosomal S6 protein (phospho-S6 or P-S6), a marker for enhanced mTOR signaling. We find P-S6 expression in cortex as well as subcortical regions. We have defined mTOR activation in fetal TSC brain tissue. Single cell mutational analysis of these regions reveals somatic mutations suggesting that even though these lesions are distinct from tubers, they arise by biallelic gene inactivation. We have generated two new in vitro TSC models and have identified several new proteins that are upregulated in TSC.

15. SUBJECT TERMS
tuberous sclerosis, mTOR, somatic mutation, ribosomal S6 protein

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Introduction

Tuberous sclerosis complex (TSC) is an autosomal disorder resulting from mutations in the TSC1 or TSC2 genes that is associated with epilepsy, cognitive disability, and autism. TSC1/TSC2 gene mutations lead to developmental alterations in brain structure known as tubers in over 80% of TSC patients. Loss of TSC1 or TSC2 function in tubers results from biallelic TSC gene inactivation and leads to activation of the mTOR cascade as evidenced by phosphorylation of ribosomal S6 protein (P-S6). Several new findings warrant further investigation of the mechanisms through which TSC gene mutations lead to developmental alterations in brain structure. Recent MRI studies suggest that there are subtle widespread abnormalities in TSC brains that contribute to neurocognitive deficits and *in vitro* evidence suggests that reduction of Tsc1 in rat neurons leads to altered dendrite structure.

First, we proposed to define subtle structural alterations distinct from tubers in post-mortem TSC brain specimens in the cortex, thalamus, basal ganglia, and cerebellum which may contribute to epilepsy, infantile spasms, and neurocognitive abnormalities in TSC using neuronal and astrocytic protein markers. Then, we hypothesized that P-S6 is expressed in these non-tuber brain lesions as well as tubers reflecting mTOR cascade activation similar to tubers. Next, we proposed to identify somatic second hit mutations in single microdissected P-S6 labeled cells in non-tuber brain lesions as a strategy to define whether all structural abnormalities in TSC require biallelic TSC gene inactivation. We have sought to determine whether P-S6 labeled giant cells in tubers and non-tuber brain lesions express a single or multiple somatic second hit mutations to test the hypothesis that structural lesions form by a clonal cellular expansion. We have recently generated two new *in vitro* model systems to study TSC. We have identified stem cell marker proteins that provide insights into lesion formation in TSC. As a direct consequence of the CDMRP funding we have defined mTOR activation in fetal TSC brain tissue. During the two-year funding period, we have made strides in accomplishing all of the proposed goals. We have presented our work at national and international meetings, we have three papers published and one in press that summarizes our work.

Body

Clinical Features

The tuberous sclerosis complex (TSC) is an autosomal dominant disorder affecting children and adults resulting from mutations in one of two genes, *TSC1* (TSC1) or *TSC2* (TSC2). TSC is estimated to occur in 1:8000 live births (O'Callaghan et al., l998). TSC affects multiple body organ systems including the heart, kidney, skin and eye. However, the most disabling manifestations of TSC reflect abnormalities in brain function. For example, epilepsy occurs in over 70-80% of TSC patients and infantile spasms, a devastating epilepsy syndrome often associated with profound mental retardation and dismal neurological prognosis, occurs in 20-30% of babies with TSC (Sparagana and Roach, 2000). Comorbid neuropsychological disorders such as autism, mental retardation (MR), pervasive developmental disorder, attention deficit disorder (ADD), and obsessive-compulsive disorder (OCD) are common in TSC patients (Prather and de Vries, 2004). Thus, TSC is a common cause of significant and disabling neurological, cognitive, and behavioral disorders in children and adults.

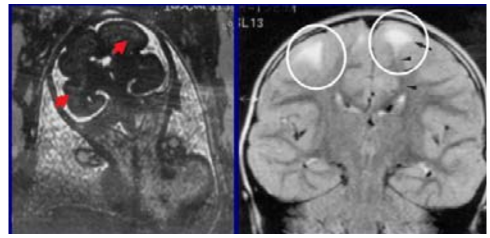

Figure 1. Left, Fetal brain MRI depicting two tubers at 25 weeks gestation (arrows). Right, two tubers in mature brain (circled).

Neuropathological Features

The neurological manifestations of TSC are believed to result from structural abnormalities in the brain that form as a consequence of TSC gene mutations. Tubers (Figs.1 and 2), present in over 80% of pediatric or adult TSC patients, are focal developmental abnormalities of cerebral cortical cytoarchitecture that are characterized histologically by disorganized cortical lamination and the presence of cells with aberrant morphologies such as dysplastic neurons (DNs), large astrocytes, and a unique cell type known as giant cells (GCs; Huttenlocher and Wollman, 1991; Crino and Henske, 1999). Tubers are single or multiple lesions detected by neuroimaging that form during embryogenesis. Tubers have been identified in fetal life as early as 20 weeks gestation (Fig.1). In older children and adults, tubers frequently calcify. Tubers are believed to be an important cause of epilepsy in TSC and for many patients who do not respond to AEDs, surgical resection of a tuber is necessary to achieve seizure control (Koh et al., 2000).

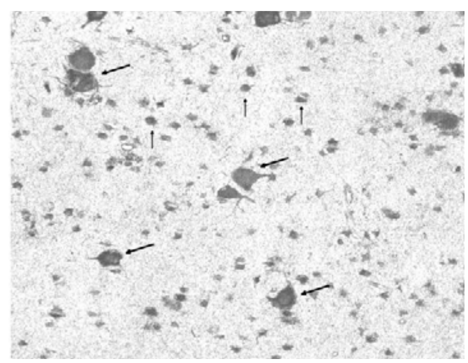

Figure 2. Tuber probed with MAP2 antibodies. GCs (large arrows) are distributed from the pial surface to the subcortical white matter without clear radial or laminar orientation and they may appear in clusters or lines. DNs are smaller (small arrows) and interspersed with GCs.

However, in the few reported neuropathological analyses of the post-mortem TSC brain, disruption of normal brain architecture distinct from tubers including small structural abnormalities including heterotopias, subcortical nodules, radial migration lines, areas of hypomyelination, and small cortical dysplasias. These

lesions differ from tubers in that they are smaller, GCs are an infrequent finding, cortical lamination is mildly altered, and they do not exhibit calcification, Recent MRI analyses in TSC patients have confirmed subtle structural abnormalities outside of tubers in the cortex and within subcortical structures such as the thalamus and basal ganglia (Ridler et al., 2001) and suggest that these non-tuber brain lesions, in addition to tubers, may contribute to autism and cognitive disability in TSC. The histopathology of these lesions has not been comprehensively investigated and the mechanistic relationship of these abnormalities to TSC gene mutations is unknown i.e., do these lesions form by similar processes as tubers, are they secondary events, or are they a unique phenotype of TSC? In addition, while activation of the mTOR cascade is a robust finding in tubers, it is unclear whether mTOR activation occurs in non-tuber lesions. Moreover, a compelling observation is that some TSC patients exhibit profound neurological disorders i.e., infantile spasms or autism, but have **normal** neuroimaging studies. Likely, there are microscopic structural alterations not detectable by MRI that can disrupt neurological function. Thus, an important new perspective on neurological manifestations of TSC is to fully consider the effects of radiographically visible lesions (tubers) as well as radiographically minimal or occult lesions on brain function.

mTOR Activation and Biallelic TSC Gene Inactivation

Mutations in *TSC1* or *TSC2* likely have a significant impact on neuroglial development (see Orlova and Crino 2010). TSC1 and TSC2 form a functional protein-protein heteromeric complex that constitutively inhibits the activation (phosphorylation) of mTOR (mammalian target of rapamycin), p70-S6-kinase, and ribosomal S6 proteins (Fig.3) that contribute to ribosomal assembly and protein translation (Arrazola et al., 2002; Kenerson et al., 2002). The mTOR cascade (Fig. 3) functions downstream of the insulin-like growth factor-1 (IGF-1) receptors, PI3K, and Akt and serves as a key regulator of cell size via effects on ribosome biosynthesis and 5'-cap dependent mRNA translation (Schmelzle and Hall, 2000; McManus and Alessi, 2002). Constitutive negative modulation of this cascade by TSC1-TSC2 results in growth suppression, diminished protein synthesis, and restricted cell size. However, in response to growth factor stimulation e.g., IGF-1, nutrient availability, or stress, TSC2 is inactivated via Akt-mediated phosphorylation and causes Rheb (Ras homolog expressed in brain) mediated phosphorylation (activation) of mTOR, p70S6 kinase, ribosomal S6, and 4EIBP.

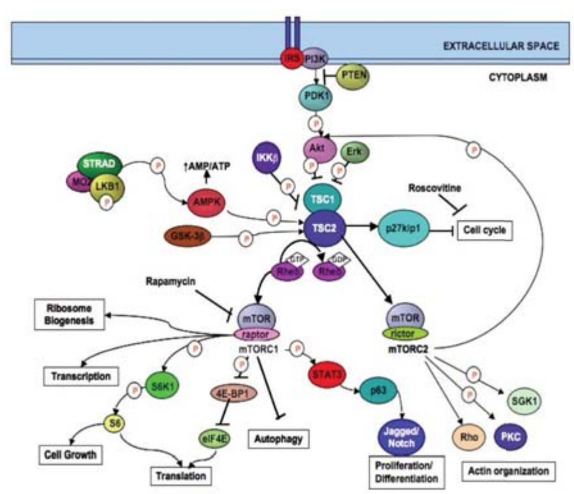

Figure 3. The mTOR cascade with TSC1 and TSC2 functioning as central upstream regulators.

In TSC lesions, loss of TSC1 or TSC2 function leads to mTOR cascade activation and aberrant phosphorylation of ribosomal S6 protein (P-S6; Tee et al., 2002; Inoki et al., 2002). In keeping with the Knudsen "two-hit" mutational model, inactivation of both TSC1 or TSC2 alleles is necessary for mTOR activation and lesion formation (Green et al., 1994; Henske et al., 1999). By this mechanism, a somatic "second hit" mutation superimposed on an

existing germline mutation leads to loss of TSC1 or TSC2 function. Phosphorylation of ribosomal S6 protein is increased in subependymal giant cell tumor specimens from TSC patients (Chan et al., 2004) that exhibit biallelic inactivation. We have reviously demonstrated (Baybis et al., 2004) cell specific activation of the mTOR cascade in giant cells in human tubers as evidenced by P-S6 expression. Our lab was the first to demonstrate that expression of phospho-ribosomal S6 (P-S6) protein is a robust marker for cells lacking TSC1 or TSC2 function in tubers.

Key Research Accomplishments

The mission of our ongoing funding cycle based on the proposed Statement of Work has been to define how changes in brain structure result from alterations in TSC gene function. Over the past year we have optimized strategies for single cell microdissection, single cell gene mutation analysis, and morphometric analysis of post-mortem TSC brain tissue (Crino et al., 2010; Marcotte et al., 2012). We have demonstrated aberrant expression of growth factors and their cognate receptors in TSC (Parker et al., 2010). In addition, we have developed two new models for TSC using both *in vitro* and *in vivo* techniques that allow us to assay changes in gene and protein expression following TSC gene knockdown (Tsai et al., in press).

Single Cell Gene Mutational Analysis

We have previously demonstrated that we can define germline and somatic *TSC1* or *TSC2* mutations in single microdissected P-S6 labeled cells. These experiments demonstrated for the first time the mutational mechanisms that lead to tuber formation and provide a novel strategy that can be applied to **defining the spectrum of germline and somatic second hit mutations in tubers** and non-tuber brain lesions. These results also allowed us to provide evidence for the first time for a somatic second hit model for tuber formation during brain development in which a progenitor cell sustains a somatic “second hit” mutation early in corticogenesis continues to divide, and generates progeny lacking functional *TSC1* or *TSC2* (Crino et al., 2010). Our results, which were directly funded by this DOD initiative, define that a somatic second hit mechanism very likely is responsible for tuber formation. As a consequence, the mTOR cascade is activated, leading to cytomegaly and perhaps, impaired migration or lamination. Tubers form as a mosaic lesion of null cells containing germline and somatic TSC gene mutations (in red) and haploinsufficient cells (e.g., dysplastic neurons, depicted in blue), containing only germline mutations. Interestingly, in this model the genotype of cells in adjacent non-tuber cortex (depicted in blue to the right of the tuber) is the same as dysplastic neurons (also in blue) within the tuber.

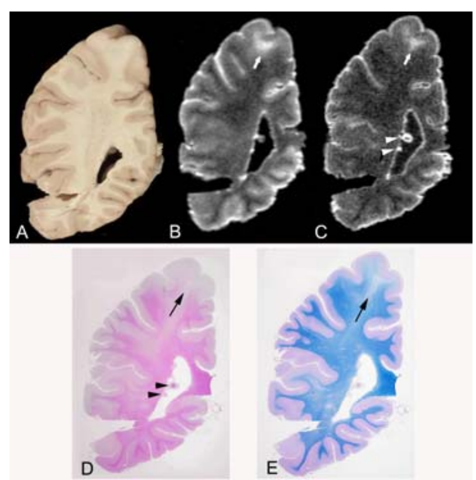

Figure 4. Multiple areas of structural abnormalities (D,E) not detected on post-mortem gross pathology (A) or tissue MRI (B,C).

There are several unresolved issues relating to structural abnormalities and TSC1/TSC2 function in the developing brain. For example, an important unanswered question is whether tubers are formed by a single somatic mutation or by multiple

second hit mutations. A logical next question is whether somatic mutations occur at a developmental critical period and whether they occur simultaneously in a "shower" of mutational events. These notions have obvious importance for the realistic development of *in utero* therapy to prevent tuber formation (discussed in Tsai et al., in press).

Selective Activation of mTOR Pathway in Non-Tuber Lesions

A new direction in understanding the broad picture of neurological dysfunction in TSC is to define to what extent there are cytoarchitectural abnormalities in **non-tuber brain** areas**.** Based on our preliminary data, we propose that there are subtle structural alterations distinct from tubers that may be not be seen by MRI. We have completed experiments that define P-S6 expression in non-tuber brain areas in 5 post-mortem TSC cases. Our data represent analysis of the largest post-mortem TSC brain sample to date. These tissues are a precious resource and have been carefully assembled because they share many important phenotypic similarities. All patients had infantile spasms, intractable epilepsy, and significant cognitive disability. Formal IQ testing was not performed but all 5 patients were consigned to institutional living with full management of daily living activities.

P-S6 expression by immunohistochemistry was examined in non-tuber cortical regions from five post-mortem TSC brain specimens (Marcotte et al., 2012). In these cases post-mortem MRI defined only a few of the most overt brain lesions (Fig.4). P-S6 immunoreactivity identified numerous regions of aberrant cortical lamination in areas that were histopathologically distinct from tubers. In these areas, we found 1) small islands of GCs (we term these "microtubers"; Fig.5) that express P-S6; 2) only one or two GCs (expressing P-S6) surrounded by multiple P-S6 labeled dysplastic neurons ("dysplasias") or 3) heterotopia (abnormal collections of cells in subcortical white matter). These data suggest a potentially highly relevant mechanism in which non-tuber lesions may result from enhanced mTOR cascade activation and loss of *TSC1* or *TSC2* in the absence of tuber formation. It is thus possible that other brain areas may contain cells that lack functional *TSC1* or *TSC2* and yet do not form tubers, perhaps due to their embryological origin or progenitor cell subtype.

Increased P-S6 protein labeling serves as a valuable marker for aberrant mTOR activation in cells lacking *TSC1* or *TSC2*. These data raise several pivotal questions:

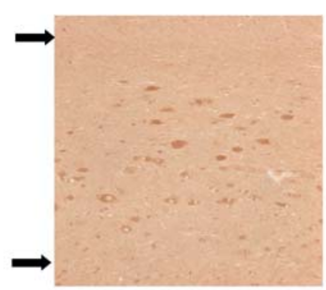

Figure 5. Cluster of P-S6 labeled GCs ("microtuber") in subcortical white matter. Arrows depict boundary of overlying normal cortex above, and white mater, below.

1) Does P-S6 expression in these cells result from biallelic gene inactivation? Ongoing analysis in the lab has revealed missense mutations in two non-tuber brain areas consistent with biallelic inactivation as a molecular cause for mTOR activation in these areas.

2) If so, then why are these lesions distinct from tubers? We don't yet understand why some lesions are tubers while others are more subtle structural abnormalities. We are embarking on further genotype analysis to define mutations in other non-tuber brain areas.

3) What are the distinct mechanisms that determine formation of tubers versus more subtle structural abnormalities e.g., loss of TSC1 or TSC2 in specific

embryonic brain regions or at specific developmental epochs or in a specific subset of progenitor cell types? Perhaps there are subsets of progenitor cells that are incapable of tuber formation or alternatively, perhaps tubers can form only at precise developmental epochs. Further studies using the methods proposed in this funding initiative are ongoing in my laboratory.

4) Are additional pathways (i.e., MAPK, which function in parallel with mTOR activated by loss of TSC1/TSC2), responsible for altered structure? These experiments are in progress in the lab. Previous work from our lab has revealed enhanced MAPK phosphorylation in tubers so a similar mechanism may function in non-tuber brain areas.

Our ongoing work will include a comprehensive analysis of TSC gene mutations in P-S6 labeled cells in non-tuber brain areas as a strategy to define the mutational spectrum of cells in non-tuber brain areas. We are also in the process of characterizing the localized expression of other kinases within or related to the mTOR cascade that may be aberrantly activated in TSC. We recently analyzed the neuropathological findings of a 32-year-old patient with a germ-line mutation in the *TSC2* gene. Post-mortem MRI combined with histology and immunocytochemical analysis was applied to demonstrate widespread anatomical abnormalities of gray and white matter structure. TSC brain lesions were analyzed for loss of heterozygosity (LOH) on chromosome 16p13. The neuropathological supratentorial abnormalities were represented by multiple subependymal nodules (SENs) and cortical tubers. In addition to cerebral cortical lesions, cerebellar lesions and hippocampal sclerosis were also observed. Immunocytochemical analysis of the TSC brain lesions confirmed the cell-specific activation of the mTOR pathway in cortical tubers, SENs and cerebellum, as well as differential cellular localization of hamartin and tuberin, the *TSC1* and *TSC2* gene products. Examination of the pathological brain regions revealed activated microglial cells and disruption of blood-brain barrier permeability.

Stem Cell Markers in TSC Brain Lesions

We have recently found that there is dramatic expression of several neural stem cell markers in TSC including Sox2 (Fig.6), Oct4, and Nanog (Orlova et al., 2010). These findings confirm earlier data demonstrating exression of nestin and collapsin response mediator protein-4 (CRMP-4) (Lee et al. 2003) in TSC brains. Ongoing studies are underway to define how these proteins are regulated and whether they lead to cell turnover in TSC brain lesions.

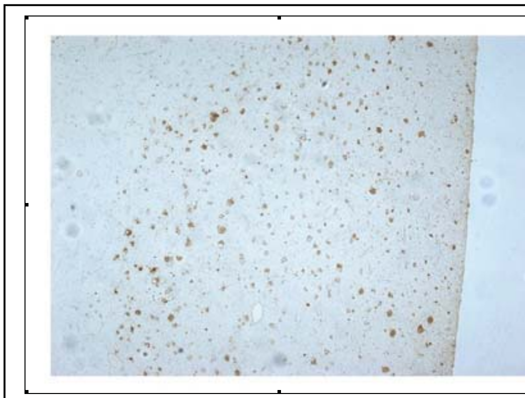

Figure 6. Sox2 expression in tuber specimen.

New Signaling Molecules

We have identified increased expression of epidermal growth factor (EGFR) and vascular endothelial growth factor (VEGF) in tubers and subependymal giant cell astrocytomas (Parker et al., 2010). Increased expression of these proteins is also detected in the Tsc1GFAPcre knockout mouse and in neural progenitor cells following shRNA knockdown (see below). These data identify new potential protein targets for therapy in TSC.

A comprehensive collaborative gene array study defined several new gene classes including proinflammatory genes and adhesion molecules as highly overexpressed in tubers (Boer et al., 2010). These sudies demonstrate clear evidence for an enhanced inflammatory response in tubers and suggests many new target molecules for TSC treatment.

New *In Vitro* Models of TSC

We have recently optimized shRNA techniques to knockdown Tsc1 or Tsc2 levels in mouse neural progenitor cells (mNPCs) and in embryonic mouse cortex (Tsai et al., in press). First, transfection of Tsc2 shRNA into mNPCs leads to enhance phosphorylation of S6 and 4-EBP1 proteins as a consequence of mTOR activation. Second, we have optimized the protocol of *in utero* electroporation (IUE) to introduce shRNA into dividing embryonic cells. Following IUE, we can visualize GFP positive cells in the embryonic cortex. This approach provides a strategy to model tuber formation in rodent brain.

Key Research Accomplishments

Boer et al., 2010
-accomplished comprehensive analysis of gene expression in tubers and defined cell inflammation and proinflammatory cytokine genes as new therapeutic targets.

Crino et al., 2010
-defined somatic second hit TSC gene mutations in tubers.

Orlova et al., 2010
-identification of SOX2, c-myc, Oct-4 and other stem cell markers in TSC lesions.

Parker et al., 2010
-identification of altered expression of EGF, EGFR, HGF, and VEGF in TSC brain lesions.

Marcotte et al., 2012
-comprehensive analysis of 5 post-mortem TSC brains with identification of phospho-S6 labeled cells in brain areas distinct from tubers in post-mortem TSC brain tissue.

-identification of subtle dysplasias and morphological abnormalities in non-tuber brain regions including subcortical areas.

-identification of isolated giant cells in non-tuber brain areas.

Tsai et al., in press
-generation of two new model systems to study TSC and demonstration of fetal mTOR activation in TSC.

Reportable Outcomes

Presentations
Crino PB. “ Focal Cortical Malformations:Sequence, Signaling, and Seizures” Merritt-Putnam Symposium, American Epilepsy Society, Seattle, WA 2008

Crino PB. “mTOR, Tuberous Sclerosis and Beyond: what TSC has taught us” American Epilepsy Society Investigators Workshop, Boston, MA 2009

“mTOR Activation: Tuberous Sclerosis and Beyond”
Investigator’s Workshop, American Epilepsy Society, Boston MA
December 2009

“New Advances in Epilepsy Therapeutics”
Albert Einstein Medical Center Neurology Grand Rounds, Philadelphia, PA
January 2010

“Neuroinflammation and Epilepsy: What TSC Has Taught Us”
International Meeting on Neuroinflammation, University of Milan, Italy
September 2010

“Insights into Focal Brain Malformations Associated with Epilepsy”
UMDNJ Medical Center Neurology Grand Rounds, Newark, NJ
October 2010

“Tissue Genetics in Tuberous Sclerosis Complex”
Special Interest Group (SIG) Symposium, American Epilepsy Society, Boston MA
December 2010

“Analysis of Focal Brain Malformations Associated with Epilepsy and Autism”
Children’s National Medical Center Neurology Grand Rounds, Washington, DC
December 2010

“Developmental Brain Malformations: A Spectrum Along the mTOR Cascade”
Department of Neurology/Neurosurgery Grand Rounds, Yale School of Medicine
March 2011

Publications
Boer K, **Crino PB,** Gorter JA, Nellist M, Jansen FE, Spliet WG, van Rijen PC, Wittink FR, Breit TM, Troost D, Wadman WJ, Aronica E. Gene expression analysis of tuberous sclerosis complex cortical tubers reveals increased expression of adhesion and inflammatory factors. *Brain Pathol* 2010;20;704-719

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Orlova KA, **Crino PB.** The tuberous sclerosis complex. *Ann N Y Acad Sci.* 2010 Jan;1184:87-105.

Parker WE, Orlova KA, Heuer GG, Baybis M, Aronica E, Frost M, Wong M, **Crino PB.** Enhanced epidermal growth factor, hepatocyte growth factor, and vascular endothelial growth factor expression in tuberous sclerosis complex. *Am J Pathol* 2011;178(1):296-305

Marcotte L, Aronica E, Baybis M, Crino PB. Cytoarchitectural alterations are widespread in cerebral cortex in tuberous sclerosis complex. *Acta Neuropathol*. 2012; 123(5):685-93

Conclusions –"So what?"

These data provide pivotal new insights into the pathological spectrum of disease in TSC nd provide new model systems to study. We have directly addressed and answered many of the questions posed in our grant proposal. Thus, as The identification of subtle cytoarchitectural abnormalities not detected by MRI yields clues as to why many individuals with TSC suffer from severe epilepsy or autism even when the MRI scan reveals only a solitary tuber or brain lesion. These data suggest that for many TSC patients structural lesions in the brain are widespread and pervasive and further demonstrate the severe consequences of TSC gene mutations on neurological functioning. Our data provide a compelling clinical case for early and in fact possibly in utero treatment with mTOR inhibitors such as rapamycin to prevent the effects of TSC gene mutations on brain formation. Upon completion of our proposed studies we wil have defined for the first time a comprehensive molecular-anatomic view of a neurodevelopmental disorder associated with epilepsy and autism. The generation of two new model systems permits more in-depth analysis of the developmental pathogenesis of TSC and the role of select therapies for epilepsy or lesion growth. Finally, identification of new marker proteins for brain lesions e.g., growth factors (Parker et al., 2010), stem cell proteins (Orlova et al., 2010), and proinflamatory molecules (Boer et al., 2010) in TSC provides new insights into how brain lesions form during pre- and post-natal development and again, yield clues to possible therapeutic interventions for TSC.

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